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Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer

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A retroviral vector containing the wild-type p53 gene under control of a β -actin promoter was produced to mediate transfer of wild-type p53 into human non-small cell lung cancers by direct injection. Nine patients whose conventional treatments failed were entered into the study. No clinically significant vector-related toxic effects were noted up to five months after treatment. *In situ* hybridization and DNA polymerase chain reaction showed vector-p53 sequences in posttreatment biopsies. Apoptosis (programmed cell death) was more frequent in posttreatment biopsies than in pretreatment biopsies. Tumor regression was noted in three patients, and tumor growth stabilized in three other patients.

Transformation of the normal cell to a malignant cell is causally related to the acquisition of a series of genetic lesions. Most of the currently known lesions cause either a gain of transforming function or loss of tumor suppressor function by the gene product. Despite the multiplicity of these lesions within a single cancer cell, studies have shown that correction of a single critical genetic lesion is sufficient to abrogate tumorigenicity in human cancer cells^{1,2}. This raises the possibility that correction of a single genetic abnormality in the cancer cell could mediate a therapeutic effect. Two gene families that contribute to carcinogenesis are dominant oncogenes and tumor suppressor genes^{3,4}. Tumor suppressor genes may undergo homozygous loss of function by mutation, deletion, methylation, or a combination of these. We postulated that it may be possible to directly restore expression of the p53 tumor suppressor gene and that this could suppress tumor growth *in vivo*.

The existence of tumor suppressor genes was originally hypothesized to explain the inheritance pattern of retinoblastoma⁵. It was postulated that both copies of the putative tumor suppressor gene must be eliminated or inactivated to eradicate the growth-suppressive function of the gene⁶. Theoretically, replacement of a nonfunctioning copy of the tumor suppressor gene in cells with loss of function by a functioning copy could restore normal growth and proliferation pathways. Possible mechanisms by which such a replacement could cause clinically beneficial effects include induction of tumor cell death by direct killing (for example, apoptosis) or bystander effects (killing or growth arrest of nontransduced tumor cells mediated by transduced tumor cells), induction of tumor cell dormancy, or prevention of malignant progression in premalignant cells⁷. The p53 gene is frequently rendered nonfunctional in human cancers

by mutation or deletion and is therefore a candidate gene for replacement⁸.

A retroviral expression vector was constructed to mediate efficient transduction of the wild-type p53 gene into human lung cancer cell lines so the effects of wild-type p53 expression in lung cancer could be studied in clinically relevant model systems of human lung cancer. A wild-type p53 cDNA driven by a β -actin promoter was inserted into a modified LNSX retroviral vector⁹. Restoration of the wild-type p53 gene suppressed growth of lung cancer cell lines and growth of human lung cancers in an orthotopic *nu/nu* mouse model, thus confirming previous observations that correction of a single genetic defect in the cancer cell decreases tumorigenicity^{10,11}. These studies provided the basis for a clinical trial to test the safety and antitumor efficacy of retrovirus-mediated delivery of the wild-type p53 gene to human lung cancer cells. Retroviral supernatant was directly injected into lung cancers with documented p53 mutations by using either a fiberoptic bronchoscope or percutaneous needle with radiologic guidance.

Patient characteristics

Nine male patients, median age 68 years (range, 51–73), who had a history of primary non-small cell lung carcinoma (NSCLC) were entered into the study (Table 1). Four patients with recurrent endobronchial lesions (three squamous cell carcinomas and one adenocarcinoma) were treated with bronchoscopic injections of the retroviral p53 expression vector TRp53A. Four patients with chest wall lesions (two large cell carcinomas, one squamous cell carcinoma, and one adenocarcinoma) were treated with percutaneous injections under computed tomographic (CT), three patients) or fluoroscopic (one patient) guidance. One patient had

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Table 1 Characteristics of patients and response to injections of retroviral vector ITRp53A

Pt no.	Sex	Age	Performance status (Zubrod)	Histologic	Prior treatment	Site of treatment	Route of treatment	Mutation (codon, base change, amino acid change)	Response of treated lesion (response duration in weeks)	Survived (weeks)
1	M	60	1	squamous	drainage of pleural effusion, 50 Gy lung tumor, 15 Gy brachytherapy	left mainstem bronchus	bronchoscopic	138, CTC→CTG, Leu→Val	viable tumor in pretreatment biopsy; no viable tumor at treated site by bronchoscopy, biopsy, and autopsy (17)	13
2	M	58	1	squamous	66 Gy lung tumor	right upper lobe	bronchoscopic	248, CCG→CTG, Arg→Leu	viable tumor in pretreatment biopsy; no viable tumor in 6 posttreatment biopsies at 1 month (10)	22
3	M	61	1	large cell	surgical resection, 60 Gy post-op	right upper chest wall	percutaneous by CT	245, GGC→AGC, Gly→Ser	stable by chest radiograph and CT scan (9); viable tumor in pretreatment biopsy; 3 posttreatment biopsies show no viable tumor	9
4	M	73	2	adenocarcinoma	vinorelbine, mitomycin, 2 months	left anterior chest wall	percutaneous by fluoroscopy	249, AGC→ATG, Arg→Met	inevaluable	3
5	M	56	2	adenocarcinoma	resection of solitary brain metastasis with whole-brain radiation, vinorelbine, mitomycin, methotrexate, 6 months; paclitaxel, 3 months; 30 Gy to lung tumor 9 months before entry	right upper lobe	bronchoscopic	269, frameshift insertion	>50% regression of treated endobronchial tumor with viable tumor in pre- and posttreatment biopsies (4)	4
6	M	72	1	squamous	resection of brain metastasis with whole-brain radiation, paclitaxel, 3 months; 45 Gy to lung tumor 9 months before entry	right posterior chest wall	percutaneous by CT	157, CTC→TTC, Val→Phe	progression by CT scan; viable tumor in pre- and posttreatment biopsies	13
7	M	57	1	large cell	doxorubicin, VP-16, 5-FU, 6 months; surgical resection, 61 Gy post-op; docetaxel, 2 months	left adrenal metastasis	percutaneous by CT	135, TGC→TAC, Cys→Tyr	stable with increased lucency on CT scan suggestive of tumor necrosis and relief of flank pain (8); viable tumor in pre- and posttreatment biopsies	22
8	M	51	1	large cell	surgical resection; doxorubicin, mitomycin & cisplatin, 9 months; 50 Gy chest wall radiation 3 years before entry	left posterior chest wall	percutaneous by CT	150, ACA→ATA, Thr→Ile 157, CTC→TTC, Val→Phe 175, CCG→AGC, Arg→Ser	stable (8); viable tumor in pre- and posttreatment biopsies	20
9	M	68	2	squamous	52 Gy lung tumor, 20 Gy right bronchus, and 67 Gy at 10 mm left bronchus	carina	bronchoscopic	145, TGG→TGA, Trp→stop	inevaluable	6

*All patients died from progression of untreated metastases or other complications.

a left adrenal metastasis from a large cell carcinoma treated with percutaneous injection under CT guidance. All patients in the study had recurrent or metastatic tumors that had progressed during prior treatment. Five patients had undergone surgical resection of either the primary lung cancer (three patients) or a brain metastasis (two). Five patients had received chemotherapy and eight patients had received radiation therapy.

Detection of vector

Polymerase chain reaction (PCR) analysis using primers specific for the retroviral transgene was used to detect the presence of vector sequences in tumor biopsies. PCR did not detect a retroviral sequence in any of the pretreatment biopsies. DNA extracted during posttreatment biopsies from two patients and post-mortem tumor from one patient showed the presence of vector-derived sequences by PCR (Table 2 and Fig. 1). Six patients showed nuclear localization of the vector by *in situ* hybridization using the neo^r probe (Table 2 and Fig. 2). Thus all patients, with the exception of patient 9, who did not com-

plete the treatment protocol, had evidence of gene transfer.

TUNEL staining of treated tumor specimens

Pretreatment and posttreatment specimens suitable for terminal deoxynucleotidyl transferase (TdT)-mediated biotin dUTP nick end-labeling (TUNEL) staining were available from seven patients. Six of the seven patients had increased TUNEL staining of posttreatment biopsies compared to the pretreatment baseline levels (Table 2 and Fig. 3).

Effects on tumor growth

Seven of the nine patients were evaluable for effects of the injection on tumor growth. Two patients were not evaluable for response, one because he died before the end of the 1-month evaluation period and one because he was unable to complete the course of treatment. Patient 4 developed aspiration pneumonia after placement of a percutaneous gastrostomy tube and died 3 weeks after the first retroviral dose. Patient 9 developed muscle weakness and respiratory distress secondary to administration of

Table 2 Assessment of gene transfer by ITRp53A retroviral vector

Pt. no.	DNA-PCR (score)	In situ hybridization* (%)	TUNEL staining*
1	+	3	17.0
2	+	3	34.0
3	+	ND†	ND
4	+	ND	27.6
5	-	2	10.2
6	-	2	10.2
7	-	2	0.8
8	-	1	23.0
9	ND	ND	ND

Results of the indicated assays on posttreatment biopsy specimens are shown. No pretreatment samples were positive in DNA-PCR and in situ hybridization assays. Examples are shown in Fig. 1-3.

*All slides were coded and read blinded by a single observer who had no knowledge of the patient or collection date. The percentage of tumor cells with punctate nuclear staining was determined in 500 cells per slide ($\times 400$ magnification). The slides were evaluated by the following scoring system: 0, no staining; 1, $<5\%$ of the cells stained; 2, 5 to 20% of the cells stained; 3, $>20\%$ of the cells stained. Slides staining ≥ 1 were considered positive; the maximum score for each patient is given; significant increases in in situ hybridization were observed in 6 of 6 evaluable cases ($P < 0.05$, sign test).

†Cells with morphologic features of necrosis were not included among the TUNEL-positive cells. The percentage of cells with nuclear staining was determined in 500 cells per slide ($\times 400$ magnification). The background level of TDT staining in pretreatment samples was $<4\%$. All slides were coded and read blinded by a single observer who had no knowledge of the patient or collection date. The maximum percent of cells staining positively is given for each patient. Mean percent \pm s.d. of TUNEL-stained cells: 1.0 ± 0.5 for all pretreatment biopsies; 10.6 ± 2.9 for posttreatment biopsies. Six of seven biopsies showed definitive increases in TUNEL staining after treatment ($P < 0.05$, two-sided Wilcoxon signed rank test).

ND, not done because of an unsatisfactory or unavailable specimen.

A general anesthetic following initial bronchoscopy, and treatments were discontinued. The patient required respirator support for 5 days and ultimately recovered.

Three of the seven patients showed evidence of tumor regression in the treated lesion. Details of the cases are as follows:

(1) Patient 1 had recurrence of a squamous carcinoma in the left mainstem bronchus at the bifurcation of the left upper and lower lobes. Bronchoscopy 1 month after retroviral injection showed marked regression of the tumor mass, and two biopsies were negative for viable tumor (Fig. 4). Three bronchoscopic biopsies in the region of the original tumor after 3 months showed no evidence of viable tumor. The patient died 4 months after treatment secondary to tumor progression at untreated sites in the left lung and distant metastases. Autopsy revealed no evidence of invasive cancer in the epithelium of the treated left mainstem bronchus.

(2) Patient 2 had an endobronchial recurrence obstructing the right upper lobe after radiation therapy. Six separate biopsies 1 month after retroviral injection showed no evidence of viable tumor at the right upper lobe orifice (Fig. 5). The patient developed progressive nodal disease in the mediastinum 2 months later.



Fig. 1 Polymerase chain reaction evaluation of vector sequences in DNA extracted from biopsies of injected tumors from patients 1 (lanes 1-4) and 3 (lanes 5-8) and an autopsy specimen of an injected tumor from patient 4 (lanes 9-12). PCR was performed with either the Apr3 + RN3 primers specific for ITRp53A sequences or p53 exon 8 primers that recognize endogenous p53 sequences as a positive control. Lanes 1, 5, 9: ITRp53A plasmid control; lanes 2, 6, 10: primer only control; lanes 3, 7, 11: pretreatment biopsy; lanes 4, 8, 12: posttreatment tumor specimen day 1, day 18 and day 5. The PCR products were probed with a 32 P-labeled, nick-translated p53 cDNA BamHI fragment.

(3) Patient 5 had tumor remaining after radiation therapy obstructing the right upper lobe orifice. Six weeks after completion of radiation therapy the patient received a single cycle of five daily endobronchial injections of the p53 retroviral vector into the tumor mass. The patient died 1 month later from distant progression and pneumonia. At autopsy, the right upper lobe orifice was patent, with $>50\%$ regression of the treated endobronchial tumor (Fig. 4).

Patient 3 had radiographic stabilization of disease for 9 weeks following a single treatment cycle. The patient died of complications related to resection of a progressively enlarging cervical vertebral body metastasis. Patient 8 had stabilization of an injected chest wall lesion for 8 weeks after two treatment cycles before a second untreated intrathoracic tumor progressed. Patient 7, after two treatment cycles, had stabilization of an adrenal metastasis for 8 weeks before the lesion progressed. In this patient, a CT scan 2 weeks after injection revealed increased lucency at the injection site, suggesting nonviable tumor representing 30% of the volume of the mass. During this time a smaller right adrenal metastasis enlarged progressively. Patient 6 had a chest wall lesion which continued to progress after the first cycle of treatment. Each of these patients had other sites of disease not treated by gene replacement injection that continued to progress during treatment with the vector. In each patient, progression at untreated sites was evident at each monthly evaluation, contrasting with the stabilization or regression noted in the treated lesions.

The degree of inflammatory cell infiltrate showed no consistent changes from the pretreatment biopsies to the biopsies taken after injection. Western blot analysis of the posttreatment serum from six of the patients did not show the presence of antibodies to retroviral proteins (data not shown).

Toxicity

There were no toxic effects directly attributable to the vector. Three patients had complications related to the procedures involved in administering the vector. Patient 1, who was treated under general anesthesia, had transient fever and required intubation for several hours after the first bronchoscopy. Subsequent procedures were done under topical anesthesia without complications. Patient 7 had a pneumothorax during CT-guided

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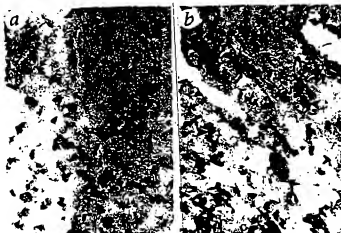


Fig 2. *In situ* hybridization with neg of pretreatment and posttreatment tumor biopsies from patient 1. a, Pretreatment biopsy; b, 24-h posttreatment biopsy; probe: LNSX plasmid. Arrows point to examples of punctate nuclear staining. Additional negative controls not shown include saline and pBR plasmid. Magnification, $\times 1000$.

Injection of an adrenal metastasis. The pneumothorax was small and did not require a thoracostomy tube. Patient 9 required intubation during the first bronchoscopic injection because of difficulty with ventilation. Subsequently, the patient required mechanical ventilation and prolonged intubation. It was believed that the patient's pulmonary function was not adequate to tolerate subsequent bronchoscopies, and the patient was removed from the protocol. He was extubated after 5 days and discharged from the hospital.

Safety

All patients had lymphocytes and sputum samples collected for up to 3 months posttreatment. Three patients (1, 4 and 5) underwent autopsy at 17, 3 and 4 weeks following entry into the protocol. Primers specific for vector sequences were used in the PCR analysis of DNA extracted from lymphocytes and sputa of patients before, during and after treatment. None of the nontumor tissues analyzed by PCR, including lymphocytes, tracheal mucosa, brain, uninjected lung, gastrointestinal tract tissues, skeletal muscle, heart, spleen, liver and testes, showed retroviral sequences, although the tissues had intact DNA as shown by amplification of endogenous *p53* exon 8 in each sample and retroviral sequences from the injected tumor of patient 4 (data not shown).



Fig 3. Staining by TUNEL technique of pretreatment (a) and 24-h posttreatment (b) biopsies from patient 1. Additional positive and negative controls not shown include H1299 *p53* homozygously deleted human NSCLC tumors grown in *nu/nu* mice injected with adenoviral vector AdCMV-*p53*, which expresses wild-type *p53*, and all sections processed in the absence of TdT. Arrows outline region of positive nuclear staining. Magnification, $\times 400$.

Discussion

Rationale and preclinical studies. Preclinical studies in *nu/nu* mice bearing tumors derived from human NSCLC cell lines showed that regional administration of viral vectors expressing wild-type *p53* prevents growth of tumors with *p53* mutations in orthotopic tumor models and mediates regression of large established tumors^{11,12}. These studies indicate that retroviral gene transfer into tumors following direct injection may occur at levels sufficient to mediate clinically significant tumor regression. Retroviral gene transfer is more efficient in cancer cells than was anticipated from studies in normal tissues¹³. The selectivity of proviral integration events for proliferating cells favors integration by tumor cells. Fresh human cancers have a relatively high fraction of proliferating tumor cells^{14,15}. Viral vectors spread readily through three-dimensional cancer cell matrices, as determined by studies with spheroids and in solid tumors in *nu/nu* mice^{16,17}.

The observation that correction of a single genetic lesion in human cancers with multiple genetic lesions can cause regression of the tumors was critical to the development of this therapeutic approach^{18,19}. Restoration of wild-type gene function in a single critical pathway appears sufficient to initiate apoptosis or G₁ arrest, resulting in either tumor regression or inhibition of tumor growth.

Efficiency of gene transfer. The presence of the transgene was detected in some but not all tumors by DNA PCR, with the longest duration appearing in an autopsy specimen 3 weeks after treatment. *In situ* hybridization provided additional evidence for uptake and nuclear localization of the vector by tumor cells.

Although direct demonstration of gene expression was not documented by reverse transcriptase PCR (data not shown), the observation of an increase in TUNEL staining indicative of apoptosis in posttreatment tumor biopsies provides indirect evidence for gene expression. Expression of wild-type *p53* in tumor cells with mutant *p53* can mediate apoptosis^{20,21}. The increase in apoptosis seen in posttreatment biopsy specimens suggested that the expression of wild-type *p53* in tumors lacking *p53* function can mediate apoptosis in human tumors *in vivo*, as has been shown *in vitro* and in animal models^{11,12,19,22-24}. The rapid response of some of the tumors is not surprising, as apoptotic cell death is observed in lung cancer cells in culture within 24 hours of wild-type *p53* expression (T. Timmons and J.A.R., unpublished observations). It is also possible that some of the effects seen were the result of G₁ arrest causing a decrease in the proliferation rate of the remaining tumor cells^{25,26}.

There are several possible explanations for our inability to detect retroviral sequences in all tumor biopsies. The high degree of cancer cell death in the posttreatment biopsies, and thus the absence of viable cells, may explain our inability to identify intact retroviral sequences in many of the tumor specimens. Other factors influencing detection of retroviral sequences in posttreatment biopsies include low levels of expression, induction of apoptosis, biopsies yielding low amounts of undegraded RNA, and the lower sensitivity of the reverse transcriptase PCR²⁷.

Although the efficiency of transduction of retroviruses into tumor cells is low compared to that of adenoviruses, our study indicates that it is sufficient to mediate a therapeutic effect. The *in situ* hybridization studies showed that in some regions of the tumor more than 20% of tumor cells had taken up the virus. The magnitude of the therapeutic responses seen suggests that bystander effects may have contributed to the tumor regression.

Fig 4. Pre- (a) and posttreatment (b) bronchoscopies of patient 9. a, The lesion in the stem bronchus; division of and lower lesions showed cell carcinoma (rows). b, Stem bronch following (1) sense of virus; right upper 30 days follow adenocarcinoma.

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Fig 5. Histologic (a) biopsy; squamous cell carcinoma of the six independent. Histologic (b) biopsy; squamous cell carcinoma of the six independent.

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DNA sequencing of a tumor biopsy as described previously¹⁴. All mutations were confirmed by sequencing or SSCP analysis of a second independent PCR reaction. Patients were not treated on protocol until 4 weeks after completion of systemic or local therapy.

Treatment protocol. The treatment protocol and safety studies have been described previously¹⁵. Patients underwent flexible fiberoptic bronchoscopy under topical or general anesthesia. A Stiffor transbronchial aspiration needle (21 gauge) was passed through the biopsy channel of the bronchoscope. The tumor site was injected with 10 ml of the appropriate retroviral supernatant. For endobronchial lesions, the 10-ml volume was divided equally among a central intratumoral injection site and two to five peripheral injection sites. Tissue was biopsied and debulked with the 1-mm biopsy forceps. For chest wall tumors, two or three 22-gauge Chiba needles were placed, equally spaced, into the tumor, and the 10-ml volume was divided equally among these injection sites. Patient 7, with the adrenal metastasis, had a single needle placed at a different site in the tumor each day. A neodymium:yttrium-aluminum-garnet laser was used for surface coagulation during the first day of treatment for patient 1. Viable tumor was present in subsequent biopsies during treatment. Lasers were not used at any other time during the protocol. After patient 6 entered the study, the FDA granted permission to treat patients with multiple cycles. Patients with locoregional tumors received intratumoral vector injections at multiple sites percutaneously under fluoroscopic guidance for a total of five daily injections. The maximum volume was 10 ml per injection.

Response and toxicity. All patients were evaluable for response and toxicity following one course of therapy. The toxic effects of therapy were evaluated according to the National Cancer Institute's Common Toxicity Criteria¹⁶. Response to therapy was assessed by chest roentgenogram or CT scans before each course of treatment. Patients were evaluable for response if they had received at least one course of therapy followed by an appropriate radiograph to document response. A complete response was defined as disappearance of all clinical evidence of tumor in the treated area without the appearance of new lesions for a period of at least 4 weeks. Patients evaluable for a less-than-complete response were those who still had a bidimensionally measurable tumor. In cases for which serial CT scans were available, tumor responses were determined on the basis of volumetric measurements. All measurements were made by one radiologist (D.L.). Partial response was defined as a 50% or greater reduction in the sum of the products of the diameters of the measurable tumor; a minor response was defined as a 25% to less than 50% reduction in the sum of the products of the diameters of the measurable lesion. Patients were designated as having progressive disease if they showed a 25% or greater increase in the size of their tumor or if they developed unequivocal new lesions during treatment in the treated area; they were considered as having no change if the treated tumor changed in any way that did not meet the criteria described above. The time to progression was measured from the first observation of reduction in tumor bulk until there was evidence of progressive disease.

For endobronchial lesions, the tumor bed was photographed at a specified distance before each course of therapy. The longest diameter and its perpendicular were measured. Size was reported as the product of the diameters. Endoscopic tumors that were not measurable were considered evaluable for response based on a minimum of four biopsies of the treated tumor bed.

Survival duration was measured from time of entry into the protocol. Each patient's response was reviewed by a Data Management

Committee consisting of a medical oncologist, radiation oncologist, and thoracic surgical oncologist.

Efficacy of gene transfer. PCR analyses were performed on pre-treatment and posttreatment tumor and normal tissue biopsies, peripheral blood mononuclear cells, sputa, and specimens collected at autopsy as described previously¹⁷. A nested PCR was used to amplify proviral sequences. The primers amplified junction sequences between p53 exon 3 and the β -actin promoter. The first set of primers was Rev Ex3 (5'-CAA ATC ATC CAT TGC TTG GGAC-3') and Apr2 (5'-GAC TCT ACC TGC GAG AAT AGG-3'). The second set of primers was R3 (5'-GGG GAC AGA ACC TTG TTT TCG-3') and Apr3 (5'-TGG GCT CGA GGT CGA CTC TAT-3'). Exon 8 of p53 was amplified as an internal control for the presence of DNA using primers Ex8A (5'-TTG GGA GTA GAT GGA GCC TT-3') and Ex8B (5'-ACA GAG GAA GAG AAT CTC CG-3'). Thirty-five amplification cycles were performed for each set of primers. The PCR analysis had a sensitivity of one copy in a background of 10⁵ genomes.

In situ hybridization. A *neo* gene probe was used to avoid the cross-hybridization of a p53 probe with endogenous p53 sequences. Slides were hybridized at 42 °C overnight with the digoxigenin-labeled LNSX plasmid. Negative controls for each section included saline and pBR plasmid. A positive control, human H460 NSCLC cells infected with LNSX at the single-copy level grown in *nu/nu* mice as a subcutaneous tumor, was included in each experiment. The slides were incubated with 0.2% anti-digoxigenin-alkaline phosphatase-conjugated antibody in 100 mM Tris-HCl buffer (pH 7.5). The color reaction was developed with a phosphate and polyvinyl alcohol buffer. The slides were counterstained with Giemsa stain.

TUNEL assay for DNA fragmentation. The TUNEL assay was a modification of a previously described technique^{18,19}. Slides were counterstained with 0.4% methylene green. Corresponding hematoxylin and eosin-stained slides were evaluated for the presence of an inflammatory cell infiltrate and were graded on a scale of 1 to 4.

Statistical analysis. A single-arm study design was used. To prevent enrolling more patients than necessary in the trial if excessive toxicity was found, a Bayesian early stopping rule was implemented. The Wilcoxon signed rank test and the sign test were used for comparisons before and after treatment of the percentages of cells showing TUNEL staining and *in situ* hybridization, respectively.

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